

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Docket No.: MORENO-LOPEZ

In re PATENT Application of:)
)
SONIA MORENO-LOPEZ & MARCOS TIMÓN-JIMENEZ) Examiner: Anne Marie Sabrina Wehbe
)
Appl. No.: 10/816,465) Group Art Unit: 1633
)
Filed: April 1, 2004) Confirmation No.: 8524
)
For: MEANS FOR ELICITING AN IMMUNE RESPONSE AND A METHOD THEREFOR)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER RULE 131 (a)

S I R:

Sonia Moreno-Lopez and Marcos Timon-Jimenez depose and declare as follows:

1. We are co-inventors in the above identified patent application, which entered the USA national phase of PCTDE02/03798 on April 1, 2004 claiming priority from German patent applications Nos.: 101 48 697.9 of October 2, 2001 and 101 56678.6 of November 12, 2001.

2. In an Office Action dated July 23, 2008, the Examiner in the above application rejected claim 43 as anticipated by the publication by Schirmbeck et al., J. Mol Med (2001) 79: 343-350 and published May 3, 2001 (hereinafter: "Schirmbeck reference"). Claim 42 was deemed obvious over the Schirmbeck

reference in view of a publication by Makkerh et al. (1996) Current Biology, Vol. 6(8), 1025-1027.

3. We make this declaration to establish that we conceived and actually reduced to practice the invention before the date of publication of the Schirmbeck reference as supported by the material attached hereto as Exhibits A-E.

4. Attached hereto as Exhibit A are 4 pages, of which page 1 is a protocol on the coupling of the hairpin-shaped oligonucleotide, which subsequently close the ends of the covalent MIDGE-vector with the NLS peptide. The product is designated as NLS-MOL-GGGA-NH and later ligated with the MIDGE-vector. The cross linker provided is designated as sKMUS (referred to also on page 11, line 16 in the English translation of the WO 03/031469). This page bears a date of September 18, 2000. Page 2, titled Purification... is dated October 2, 2000. Page 3 which shows the agarose control gel with the obtained NLS-MOL-GGGA-NH- fractions as referred to on page 2. Page 4 shows another control gel with MOL-GGGA-NH-, NLS-MOL-GGA-NH-and TAT-MOL-GGA-NH fractions, dated October 2, 2000. Translations provided for pages 1 and 2 are behind the pages.

5. Attached hereto as Exhibit B are 11 pages showing the dates of the HPLC runs for purifying the oligonucleotide NLS-MOL-GGGA-NH, whose synthesis is shown on pages 1-4 in Exhibit A, and was carried out on October 2, 2000,.

6. Attached hereto as Exhibit C, are two excerpts from the lab book dated October 4, 2000 showing electrophoresis gels with the fractions of the NLS-MOL-GGGA-NH oligonucleotide successfully purified with HPLC. These are hairpin-shaped oligonucleotides from the HPLC runs from the 11 pages of Exhibit B which form part of the inventive MIDGE-constructs.

7. Attached hereto as Exhibit D is a page from a production protocol dated December 22, 2000 with a translation, which evidences the synthesis of the inventive MIDGE-NLS vector (here: MOK-HBsAGSAY1-NLS-M). from the prior

Docket No.: MORENO-LOPEZ
Appl. No.: 10/616,485

synthesized and HPLC purified NLS-MOL-GGGA-NH hairpin-shaped oligonucleotides and the MOL-D-oligonucleotide. A comparable MIDGE HbsAG NLS-construct is disclosed on pages 12-13 paragraph 4 of the English translation of WO 03/031469.

8. Attached hereto as Exhibit E is a page showing the data of the production of the NLS-coupled MIDGE vectors in association with the crosslinker sKMUS. Data as entered on December 19, 2000 remain in that data base.

9. The Exhibits A-E evidence that at all relevant times, we were in possession of the MIDGE-NLS prior to the date of the Schimbeck reference and that we were diligent in filing the patent applications.

The declarants further state that the above statements were made with the knowledge that willful false statements and the like are punishable by fine and/or imprisonment, or both under Section 1001 of Title 18 of United States Code, and that such willful false statements may jeopardize the validity of this application or any patent resulting therefrom.

Date _____

SONIA MORENO-LOPEZ

Date January 22nd 2009


MARCOS TIMÓN-JIMENEZ

EXHIBIT A

Produktname: *NLS-MOL-666ANK* Chargennummer: *NLS-180900*

Datum: 18.09.00

Oligonukleotid-Kopplung mit:

Größe des Reaktionsansatzes: µg Oligos

	Name	Chargen-Nr.	Konz. (µg/µl) o. mM	
Oligos:			6,5	= 1mM
zu koppelndes Molekül:	NLS		2 mM	
Crosslinker:		BE44012	50 mM	
MW (g/mol)				

Bemerkung: Peptid und Crosslinker-Lösungen vor der Reaktion frisch ansetzen

Reaktion der Oligos mit Crosslinker:

Volumen (µl)	Substanz	Konz.	einzuwiegende Masse an Crosslinker in mg
100	MOL-GGGA-NH	6,5 µg/µl	2,40
100	sKMUS	50 mM	
200	coupling buffer (pH 7.0)	5x	
600	Wasser (Milipore)		

Inkubation: 120 min bei 37°C *1650 - 1900 → -20°C üN*
(4x je 25 µl sKMUS nach 0,30,60 und 90 min zugeben) 19.09.

Stoppen der Reaktion mit:

50 µl 1 M Tris HCl pH 7,5

1ml-Ansätze auf 3 Eppis verteilen: + 33 µl 3M NaAc, + 832,5 µl EtOH p.a.
EtOH-Fällung: *+ 6,6 µl 1M MgCl₂ 10% EtOH je 500 µl*

Volumen (µl)	Substanz
100	3 M Natriumacetat (=10 % des Ansatzes)
20	1 M MgCl ₂ (= 2% des Ansatzes)
2500	Ethanol (p.a.) (= 2,5x Vol. des Ansatzes)

Inkubation: 30 min bei -70 °C; 30 min 4 °C u. 12.000 rpm zentrifugieren
 anschließend mit 1-2 ml EtOH (70 %) waschen, 15 min 4 °C u. 12.000 rpm zentrifugieren.

Kopplung mit *NLS*

Volumen (µl) *je 270 µl in 3 Ansätze, die 3 dann poolen.*
 Pellet in (800) Wasser aufnehmen, auflösen lassen.
 Dann 100 5x couplin buffer (pH 7.0)
 100 0 (2 mM bei Peptiden) zugeben.
 Inkubation: 60 min bei 37 °C

Product Name: NLS-MOL-GGGA-NH Batch No.: NLS - 180 900

Date: 09/18/00

Oligonucleotide coupling partner:

NLS

Size of reaction batch:

650 µg Oligo Primer

	Name	Batch No.	Concentration (µg/µL) or mM	
Oligo Primer	MOL-GGGA-NH	180053	6.5	= 1 mM
Molecule to be coupled	NLS		2 mM	
Cross-linker	sKMUS	3E 44012	50 mM	
Mol. Weight (g/mol)	480,47			

Remark: Prepare peptide and cross-linker solution directly before start of reaction

in DMF 2083 µL 50 mg

Reaction of Oligo Primer with Cross-linker:

Volume (µL)	Substance	Concentration	Amount of Cross-linker in mg
100	MOL-GGGA-NH	6.5 µg/µL	2.40
100	sKMUS	50 mM	
200	coupling buffer (pH 7.6)	5x	
600	Water (Millipore)		

Incubation: 120 min. at 37°C 4:50 p.m. - 7:00 p.m. → -20°C overnight
(add 4x 25 µL sKMUS, after 0, 30, 60 and 90 min.) 09/19

Reaction termination accomplished by adding:

50 µL 1 M Tris HCL pH 7.5

1 mL batches, apportioned onto 3 reaction tubes: + 33 µL 3 M sodium acetate
+ 6.6 µL MgCl₂Ethanol precipitation:

+ 832.5 µL Ethanol (p.a.) (900 µL of 70% Ethanol)

Volume (µL)	Substance
100	3 M sodium acetate (= 10 % of reaction batch)
20	1 M MgCl ₂ (= 2 % of reaction batch)
2500	Ethanol (per analysis) (= 2.5 x Volume of reaction batch)

Incubation: 30 min. at -70°C; 30 min. at 4°C and centrifugation at 12.000 rpm, subsequently washing with 1-2 mL Ethanol (70%), 15 min. at 4°C and centrifugation at 12.000 rpm.

Coupling with NLSDissolve pellet in
then addVolume (µL)
(800)
100
100

270 mL for each 1/3 reaction batch, then pool the 3 batches

5 x coupling buffer (pH 7.0)
0 (2 mM in case of peptides)

Incubation: 60 min. at 37°C

Produktname:

Datum:

**Reinigung der Peptid gekoppelten Oligonukleotide
mittels HPLC:**

Laufsystem:

☒
☐

100 mM Ammoniumcarbonat / Acetonitril

Wasser / Acetonitril

Gradient: von 0 % Acetonitril auf 30 % Acetonitril in 50 min

Fraktionen eindampfen und in Wasser aufnehmen.**Kontrolle der gesammelten HPLC-Fraktionen:**☐
☒

20 % PAGE-Gel

4 % Agarose-Gel

Peptid-Oligonukleotid-Fraktion rein ?

☒
☐

ja

nein

Wenn: **ja**

Dann: Freigabe der Peptid-Oligonukleotide.

Wenn: **nein**Dann: erneute HPLC-Reinigung und Fällung der Fraktionen,
Kontrolle auf Agarose-Gel.

Datum/Unterschrift:

02.10.00 *F. Sal*

Product Name:

Batch No.:

Date:

**Purification of peptide-coupled oligonucleotides
by HPLC**

Mobile phase: ☒ 100 mM ammonium carbonate / acetonitrile
☐ water / acetonitrile

Gradient: from 0% acetonitrile to 30% acetonitrile in 50 min.

Fractions have to be evaporated and re-dissolved in water.**Analysis of collected HPLC fractions:**

☐ 20% PAGE gel
☒ 4% agarose gel

Peptide-oligonucleotide fraction included? ☒ yes
☐ no

If: **yes**

Then: approval of peptide-oligonucleotides

If: **no**

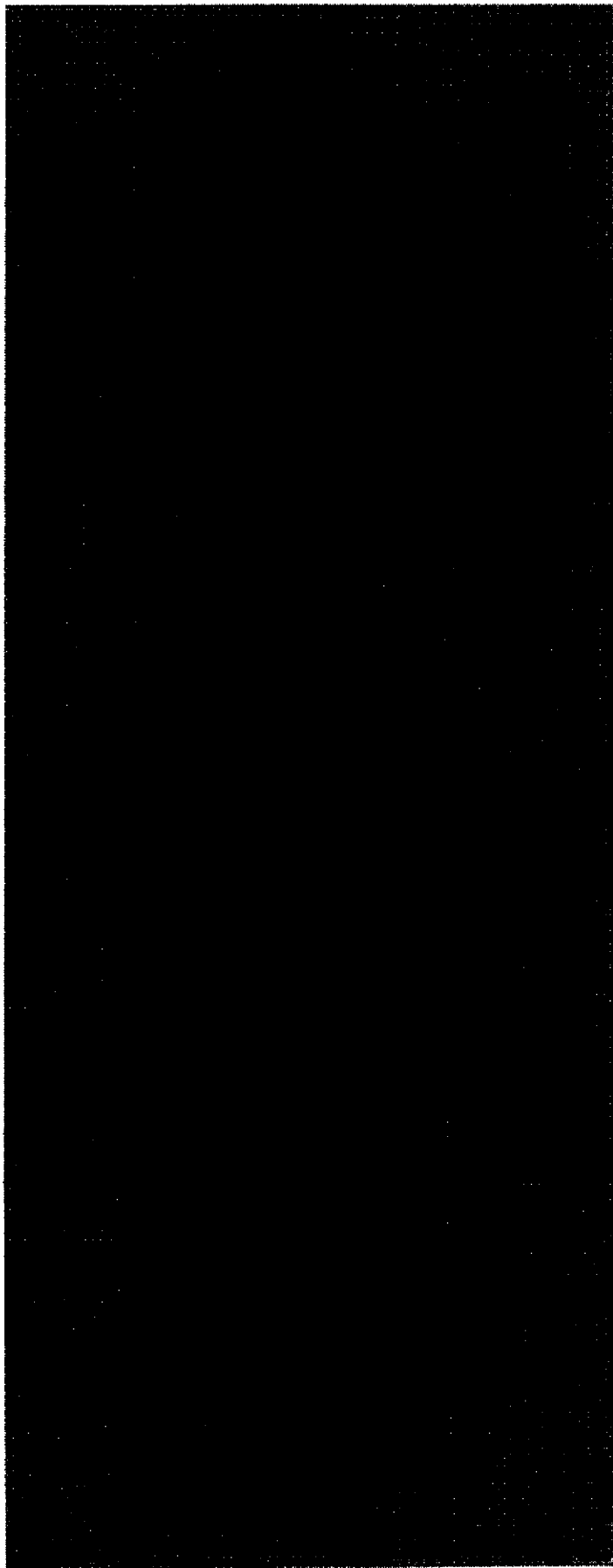
Then: a further HPLC purification and precipitation of fractions, analysis with agarose gel

Date / signature: 10/02/00

NLSTATHPLCFraktionen

NLS-780900

NLS-NBL 666 ANH



side auch LB 6 S-~~88~~-88
82-83
FS

#124 & #125, MOL-GGGA-NLS & -TAT, mel 2000-10-28

1 2 3 4

- 1 100% Mark
- 2 MOL-GGGA NH
- 3 NLS - MOL-GGGA NH
- 4 TAT - MOL-GGGA NH

EXHIBIT B

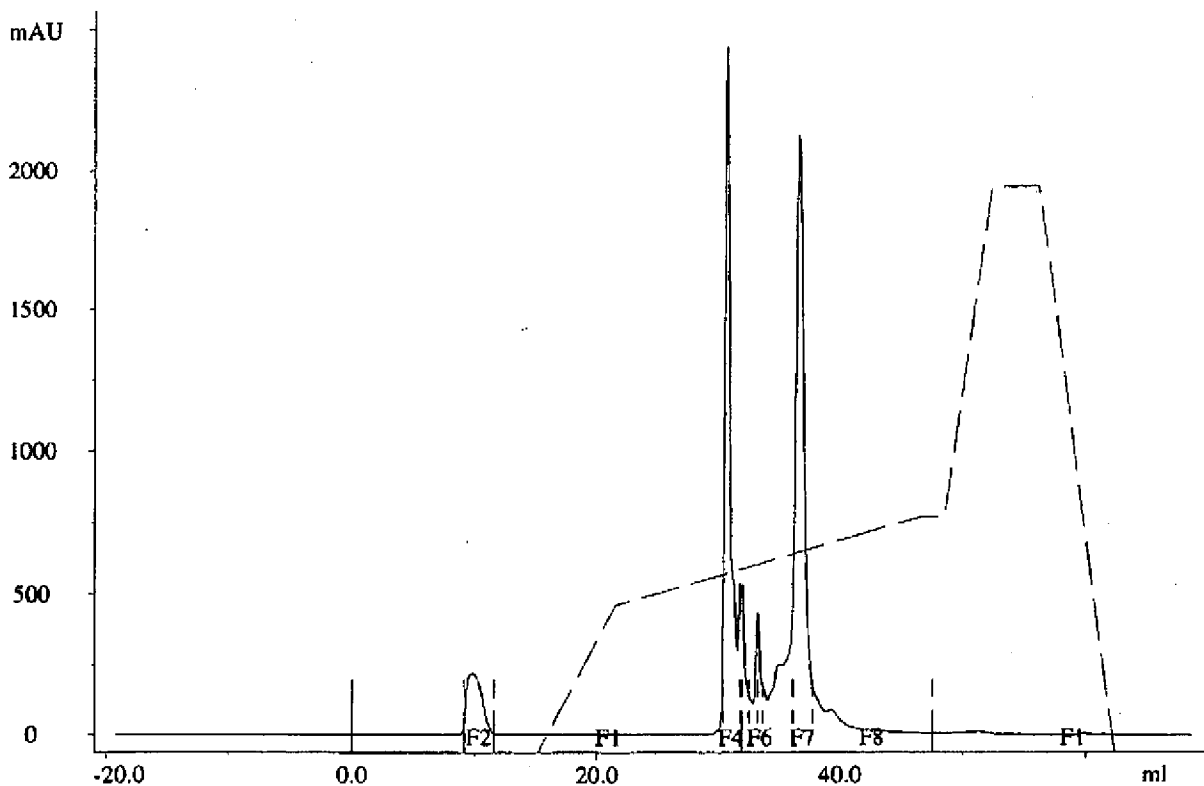
UNICORN V3.21

Run by: Florian 02.10.2000 17:32:06

Result file: c:\...\Florian\NLS180900\NLS Oligo HPLC fuer Produktion201

Method file: c:\...\Florian\NLS Oligo HPLC fuer Produktion2

----- NLS Oligo HPLC fuer Produktion201:1_UV1_260nm
----- NLS Oligo HPLC fuer Produktion201:1_Conc
----- NLS Oligo HPLC fuer Produktion201:1_Fractions
----- NLS Oligo HPLC fuer Produktion201:1_Inject



Method Information

Method name: NLS Oligo HPLC fuer Produktion201

Method created by: Florian

Date of creation: 02.10.2000 17:05:09

Target system: System 1

Method last modified by: Florian

Date of last modification: 02.10.2000 17:05:09

Strategy name: B100_100

Strategy date: 01.08.2000 14:19:12

Strategy size: 620912 bytes

UNICORN V3.21

Result file: c:\...\Florian\NLS180900\NLS Oligo HPLC fuer Produktion201

Method file: c:\...\Florian\NLS Oligo HPLC fuer Produktion2

Variables

Saeule	Nucleosil_300_C18_250x8
Flussrate	2.40 {ml/min}
Wavelength_1	260 {nm}
Wavelength_2	280 {nm}
Wavelength_3	215 {nm}
Pressure_limit	10.00 {MPa}
Equilibrate_with	1.50 {CV}
Empty_Loop_with	2.30 {ml}
Wash_column_with	1 {CV}
Target_ConcB_1	22.00 {%B}
Length_of_gradient_1	0.50 {base}
Target_ConcB_2	35 {%B}
Length_of_gradient_2	2.00 {base}
Conc_of_eluent_B	84 {%B}
Clean_with	0.60 {CV}
Reequilibrate_with	1.00 {CV}

Questions

No 1: Probenname:
NLS-MOL-GGGA-NH
No 2: Chargen-Nr. der NLS-Oligos
NLS-180900
No 3: Probenmenge:
1,5 ml
No 4: Säule:
Nucleosil-300 250x8mm
No 5: Batch-No. der Säule:
Knauer 2101s
No 6: Puffer A:
100 mM Ammoniumcarbonat
No 7: Puffer B:
80 % Acetonitril (+100mM Ammoniumcarbonat)
No 8: Bemerkungen:

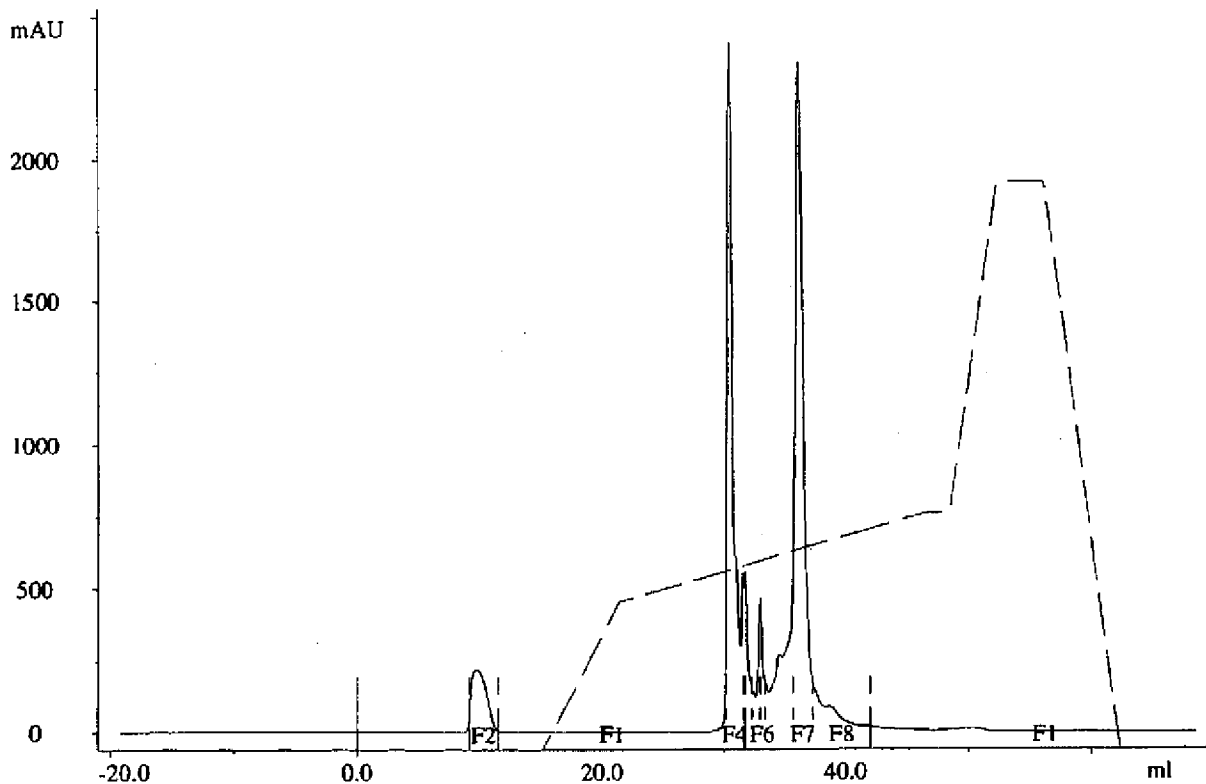
UNICORN V3.21

Run by: Florian 02.10.2000 18:10:37

Result file: c:\...\Florian\NLS180900\NLS Oligo HPLC fuer Produktion202

Method file: c:\...\Florian\NLS Oligo HPLC fuer Produktion2

— NLS Oligo HPLC fuer Produktion202:1_UV1_260nm
— NLS Oligo HPLC fuer Produktion202:1_Conc
— NLS Oligo HPLC fuer Produktion202:1_Fractions
— NLS Oligo HPLC fuer Produktion202:1_Inject



Method Information

Method name: NLS Oligo HPLC fuer Produktion202

Method created by: Florian

Date of creation: 02.10.2000 17:59:23

Target system: System 1

Method last modified by: Florian

Date of last modification: 02.10.2000 17:59:23

Strategy name: B100_100

Strategy date: 01.08.2000 14:19:12

Strategy size: 620912 bytes

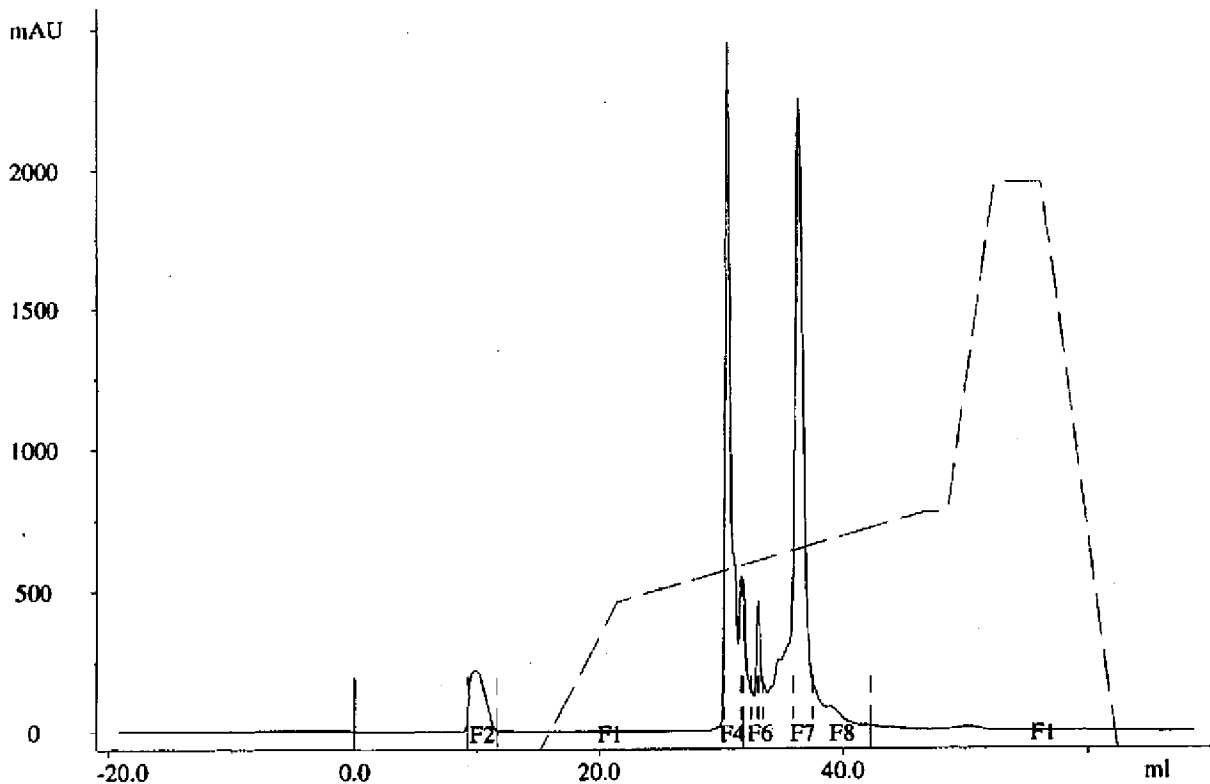
UNICORN V3.21

Run by: Florian 02.10.2000 18:49:13

Result file: c:\...\Florian\NLS180900\NLS Oligo HPLC fuer Produktion203

Method file: c:\...\Florian\NLS Oligo HPLC fuer Produktion2

—— NLS Oligo HPLC fuer Produktion203:1_UV1_260nm
- - - NLS Oligo HPLC fuer Produktion203:1_Conc
—— NLS Oligo HPLC fuer Produktion203:1_Fractions
—— NLS Oligo HPLC fuer Produktion203:1_Inject



Method Information

Method name: NLS Oligo HPLC fuer Produktion203

Method created by: Florian

Date of creation: 02.10.2000 17:59:23

Target system: System 1

Method last modified by: Florian

Date of last modification: 02.10.2000 17:59:23

Strategy name: B100_100

Strategy date: 01.08.2000 14:19:12

Strategy size: 620912 bytes

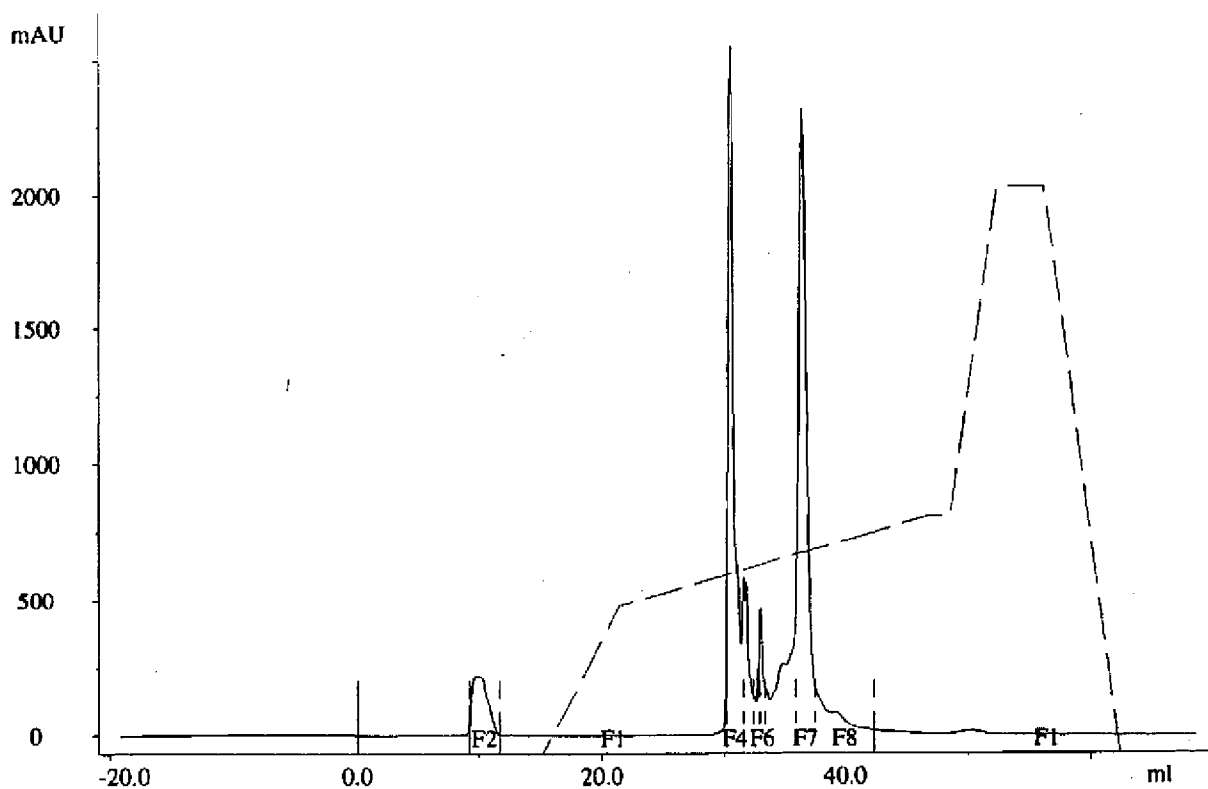
UNICORN V3.21

Run by: Florian 02.10.2000 20:10:43

Result file: c:\...\Florian\NLS180900\NLS Oligo HPLC fuer Produktion204

Method file: c:\...\Florian\NLS Oligo HPLC fuer Produktion2

— NLS Oligo HPLC fuer Produktion204:1_UV1_260nm
— NLS Oligo HPLC fuer Produktion204:1_Conc
— NLS Oligo HPLC fuer Produktion204:1_Fractions
— NLS Oligo HPLC fuer Produktion204:1_Inject



Method Information

Method name: NLS Oligo HPLC fuer Produktion204

Method created by: Florian

Date of creation: 02.10.2000 17:59:23

Target system: System 1

Method last modified by: Florian

Date of last modification: 02.10.2000 20:10:03

Strategy name: B100_100

Strategy date: 01.08.2000 14:19:12

Strategy size: 620912 bytes

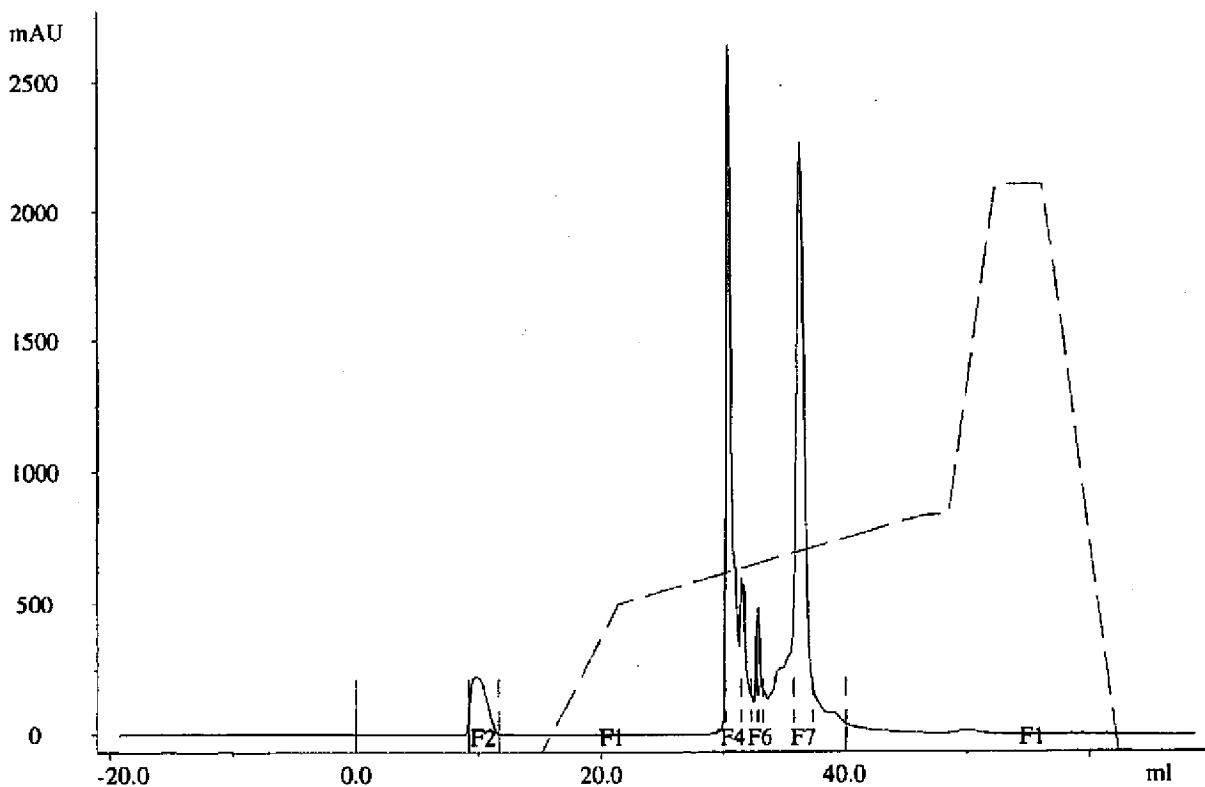
UNICORN V3.21

Run by: Florian 02.10.2000 20:48:15

Result file: c:\...\Florian\NLS180900\NLS Oligo HPLC fuer Produktion205

Method file: c:\...\Florian\NLS Oligo HPLC fuer Produktion2

—— NLS Oligo HPLC fuer Produktion205: I_UV1_260nm
- - - NLS Oligo HPLC fuer Produktion205: I_Conc
- - - NLS Oligo HPLC fuer Produktion205: I_Fractions
—— NLS Oligo HPLC fuer Produktion205: I_Inject



Method Information

Method name: NLS Oligo HPLC fuer Produktion205

Method created by: Florian

Date of creation: 02.10.2000 17:59:23

Target system: System 1

Method last modified by: Florian

Date of last modification: 02.10.2000 20:36:16

Strategy name: B100_100

Strategy date: 01.08.2000 14:19:12

Strategy size: 620912 bytes

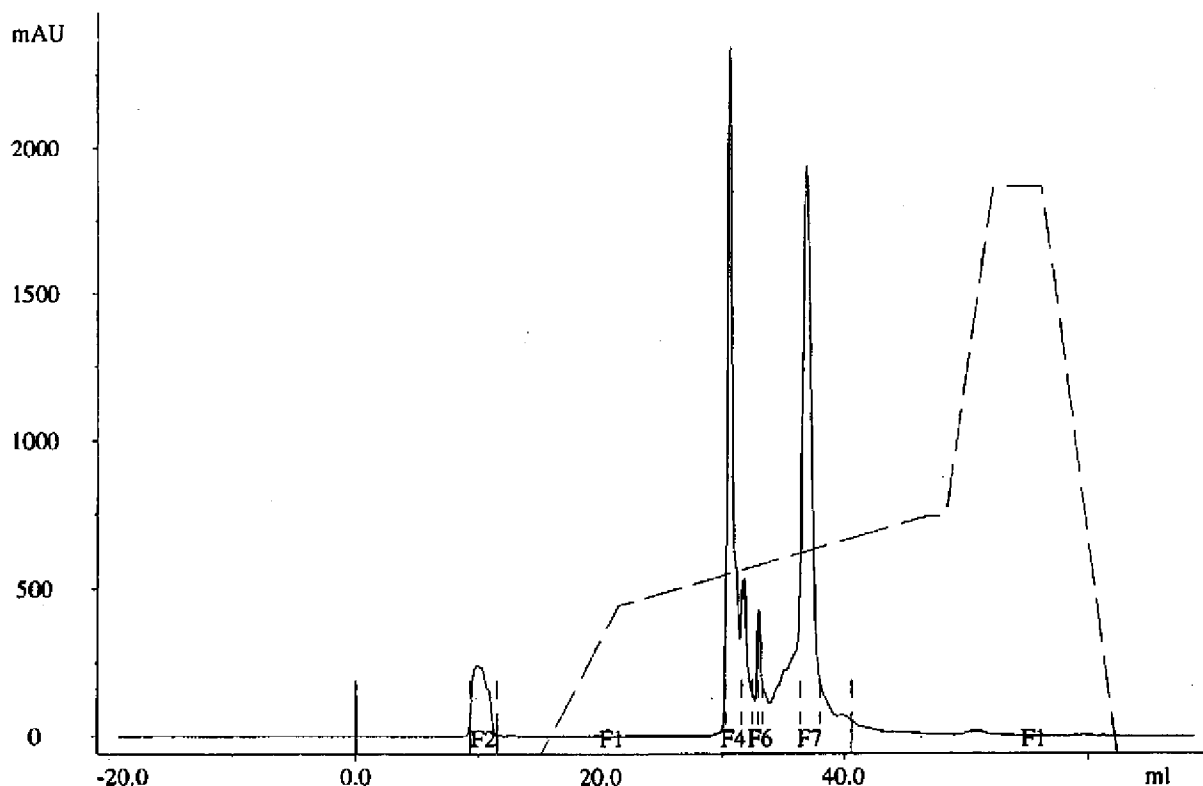
UNICORN V3.21

Run by: Florian 03.10.2000 12:12:17

Result file: c:\...\Florian\NLS180900\NLS Oligo HPLC fuer Produktion206

Method file: c:\...\Florian\NLS Oligo HPLC fuer Produktion2

----- NLS Oligo HPLC fuer Produktion206:1_UV1_260nm
----- NLS Oligo HPLC fuer Produktion206:1_Conc
----- NLS Oligo HPLC fuer Produktion206:1_Fractions
----- NLS Oligo HPLC fuer Produktion206:1_Inject



Method Information

Method name: NLS Oligo HPLC fuer Produktion206

Method created by: Florian

Date of creation: 02.10.2000 17:59:23

Target system: System 1

Method last modified by: Florian

Date of last modification: 02.10.2000 21:13:32

Strategy name: B100_100

Strategy date: 01.08.2000 14:19:12

Strategy size: 620912 bytes

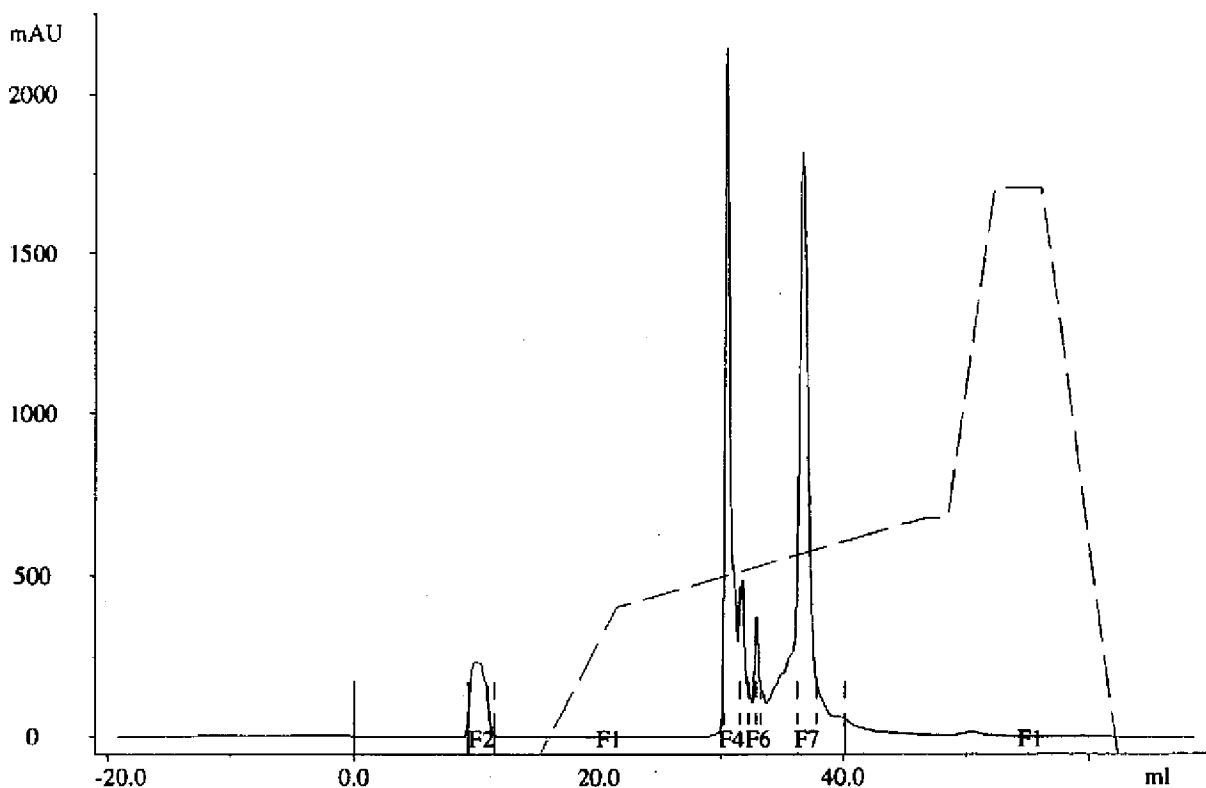
UNICORN V3.21

Run by: Florian 03.10.2000 12:49:51

Result file: c:\...\Florian\NLS180900\NLS Oligo HPLC fuer Produktion207

Method file: c:\...\Florian\NLS Oligo HPLC fuer Produktion2

— NLS Oligo HPLC fuer Produktion207:1_UV1_260nm
— NLS Oligo HPLC fuer Produktion207:1_Conc
— NLS Oligo HPLC fuer Produktion207:1_Fractions
— NLS Oligo HPLC fuer Produktion207:1_Inject



Method Information

Method name: NLS Oligo HPLC fuer Produktion207

Method created by: Florian

Date of creation: 02.10.2000 17:59:23

Target system: System 1

Method last modified by: Florian

Date of last modification: 02.10.2000 21:13:32

Strategy name: B100_100

Strategy date: 01.08.2000 14:19:12

Strategy size: 620912 bytes

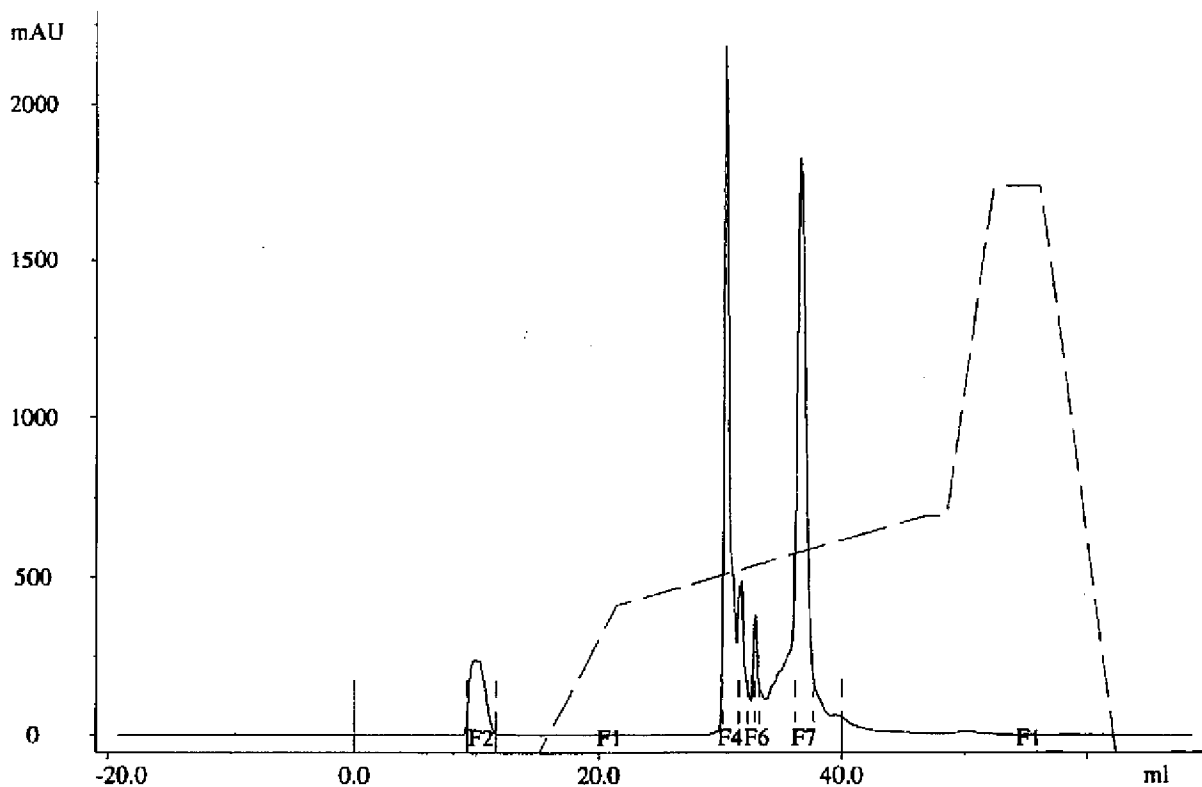
UNICORN V3.21

Run by: Florian 03.10.2000 13:27:13

Result file: c:\...\Florian\NLS180900\NLS Oligo HPLC fuer Produktion208

Method file: c:\...\Florian\NLS Oligo HPLC fuer Produktion2

— NLS Oligo HPLC fuer Produktion208:1_UV1_260nm
— NLS Oligo HPLC fuer Produktion208:1_Conc
— NLS Oligo HPLC fuer Produktion208:1_Fractions
— NLS Oligo HPLC fuer Produktion208:1_Inject



Method Information

Method name: NLS Oligo HPLC fuer Produktion208

Method created by: Florian

Date of creation: 02.10.2000 17:59:23

Target system: System 1

Method last modified by: Florian

Date of last modification: 02.10.2000 21:13:32

Strategy name: B100_100

Strategy date: 01.08.2000 14:19:12

Strategy size: 620912 bytes

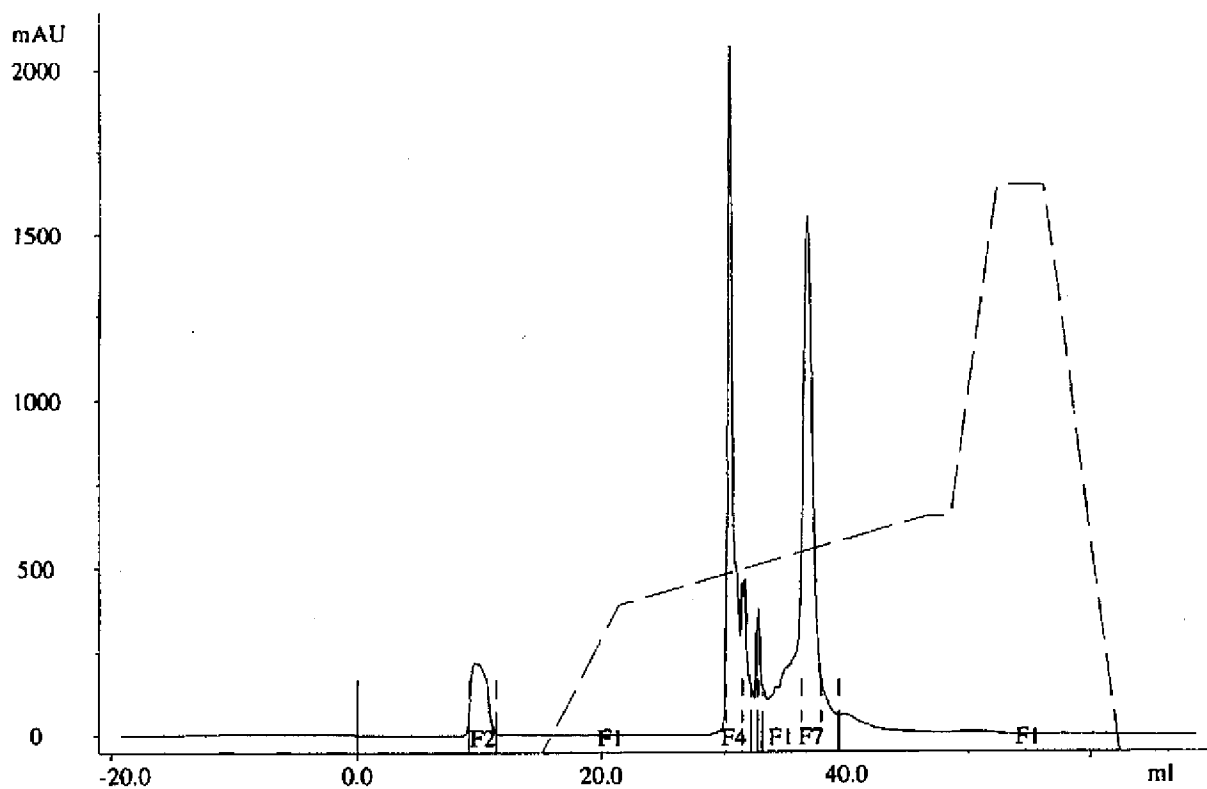
UNICORN V3.21

Run by: Florian 03.10.2000 16:10:53

Result file: c:\...\Florian\NLS180900\NLS Oligo HPLC fuer Produktion210

Method file: c:\...\Florian\NLS Oligo HPLC fuer Produktion2

— NLS Oligo HPLC fuer Produktion210:1_UV1_260nm
- - - NLS Oligo HPLC fuer Produktion210:1_Conc
- - - NLS Oligo HPLC fuer Produktion210:1_Fractions
- - - NLS Oligo HPLC fuer Produktion210:1_Inject



Method Information

Method name: NLS Oligo HPLC fuer Produktion210

Method created by: Florian

Date of creation: 02.10.2000 17:59:23

Target system: System 1

Method last modified by: Florian

Date of last modification: 02.10.2000 21:13:32

Strategy name: B100_100

Strategy date: 01.08.2000 14:19:12

Strategy size: 620912 bytes

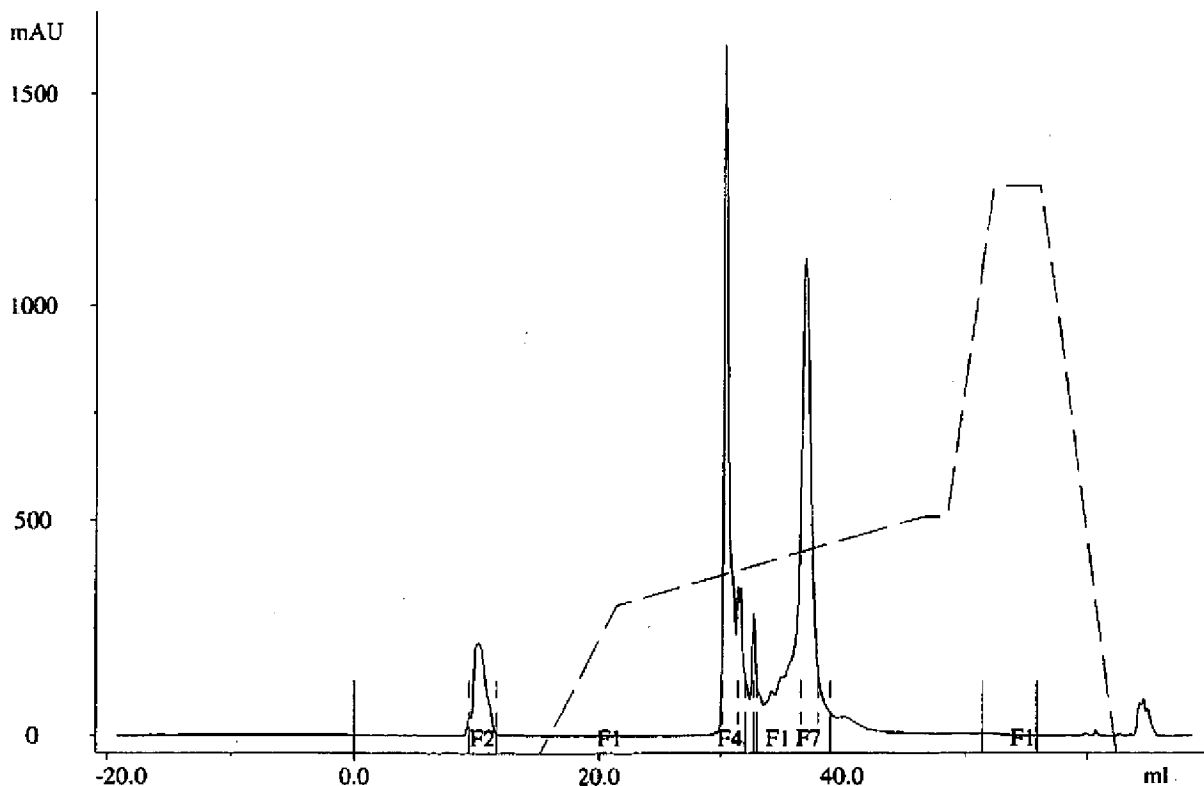
UNICORN V3.21

Run by: Florian 03.10.2000 16:48:37

Result file: c:\...\Florian\NLS180900\NLS Oligo HPLC fuer Produktion211

Method file: c:\...\Florian\NLS Oligo HPLC fuer Produktion2

—— NLS Oligo HPLC fuer Produktion211:1_UV1_260nm
- - - NLS Oligo HPLC fuer Produktion211:1_Conc
—— NLS Oligo HPLC fuer Produktion211:1_Fractions
- - - NLS Oligo HPLC fuer Produktion211:1_Inject



Method Information

Method name: NLS Oligo HPLC fuer Produktion211

Method created by: Florian

Date of creation: 02.10.2000 17:59:23

Target system: System 1

Method last modified by: Florian

Date of last modification: 02.10.2000 21:13:32

Strategy name: B100_100

Strategy date: 01.08.2000 14:19:12

Strategy size: 620912 bytes

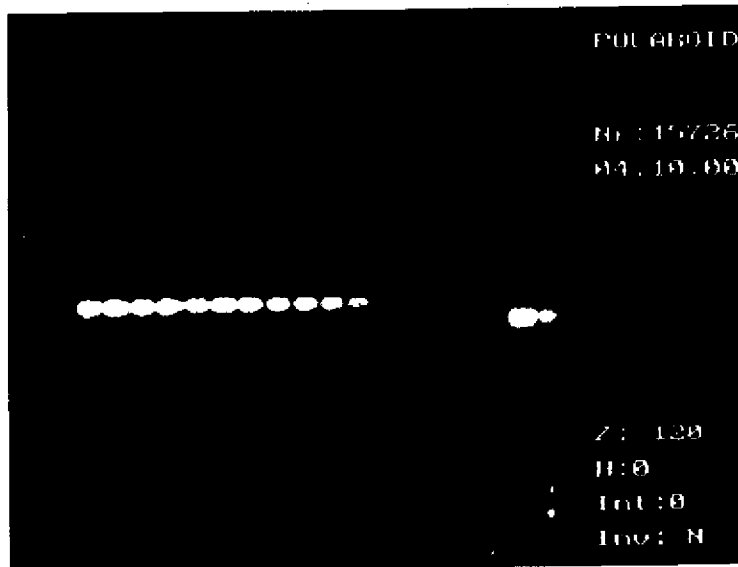
EXHIBIT C

NLS-MDL66CANH nach HPLC +TAT
je 7 µl

4.10.80

F7 ②-①① in je 50 µl
↓ TATHPLC { vor F7 50
F7! 150 µl
F8 50
nach F8 50

von HPLC-Lauf Nr.
F2 ④ 50 µl
F4 ④ 50 µl
F6 ④ 50
F vor F ④ 50
F8 ④ 50



NLSTATHPLCFraktionen

Fortsetzung von S. 82

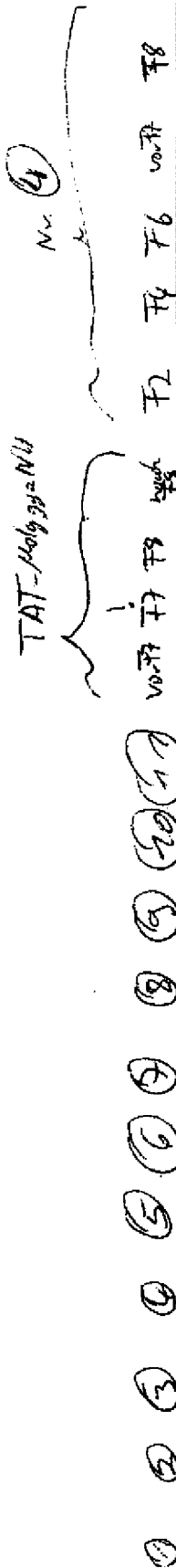


EXHIBIT D

Chargennummer:

Produktname:

251-00

MOK-HBSAGSAYAX-NLS-M

2. Ligation (SOP-P-10 Version: _____)

Berechnung der Menge an Oligos:

$$m_{\text{Oligo 1}} [\mu\text{g}] = 1370$$

$$m_{\text{Oligo 2}} [\mu\text{g}] = 137$$

 $\frac{70}{2}$ facher Überschuss $\frac{2}{2}$ facher Überschuss

	Nummer	Konzentration	Volumen [μl]	
Volumen Restriktionsverdau	----	----	51750	
Wasser (frisch entnommen)	----	----	1986	✓
Oligo 1 MOK-D	13611	5 $\mu\text{g}/\mu\text{l}$	274	✓
Oligo 2 MOK-HBSAGSAYAX-NLS	124	1,3 $\mu\text{g}/\mu\text{l}$	105	✓
ATP	187134	100mM	565	✓
Puffer G+	NL20	10-fach	471	✓
Eco 31I	61161-63	50 U/ μl	1242	✓
T4-DNA-Ligase	35114	5 U/ μl	62	✓
Gesamtvolumen		DNA: 0,55 $\mu\text{g}/\mu\text{l}$	116455	
Gefäß		---	150mm	
Datum/Unterschrift	21.12.00 M. Rother			

frühere Zugabe von
Eco 31I für 2h.
500 μl 61160, Rother
22.12.00 14:18 - 16:11

15- 20 h bei 37°C

Zeit hineingestellt (Datum / Uhrzeit): 21.12.00 / 14:02

Zeit herausgeholt (Datum / Uhrzeit): 22.12.00 / 10:03

3. Probe-T7-Polymeraseverdau (SOP-P-11 Version: _____)

	Nr.	Konzentration	Volumen [μl]	
			Ligation	T7-Verdau
Ansatz	----	----	2 ✓	2 ✓
Wasser	----	----	16,2 ✓	16 ✓
Puffer G+	NL20	10-fach	1,8 ✓	1,8 ✓
T7-Polymerase	7111	10U/ μl	-	0,2 ✓
Gesamtvolumen			20	20
Zeit [h]			2	2
Datum/Unterschrift	22.12.00 M. Rother			

1% Agarosegel: (je 5 μl auftragen)

Ligation akzeptabel?

☐ Ja☐ Nein

Probe-T7-Polymeraseverdau positiv?

☐ Ja☐ NeinWeiter mit T7-Polymeraseverdau?
(Schritt 4)☒ Ja☐ Nein

Datum/Unterschrift

Bemerkungen auf der Rückseite:

22.12.00
M. Rother

Batch No.:

251-00

Product name:

MOK-HB₅AG-SAY1x NLS-M

2. Ligation (SPO-P-10 Version: _____)

Calculation of oligo primer amounts:

m_{oligo 1} [µg] = 1370

20fold excess

m_{oligo 2} [µg] = 137

2 fold excess

	Number	Concentration	Volume [µL]	
Volume restriction digest	----	----	51750	✓
Water (freshly removed)	----	----	1986	✓
Oligo 1 M _{OL} -D	136/1	5 µg/µL	274	✓
Oligo 2 M _{OL} -GGGA-NLS	124	1.3 µg/µL	105	✓
ATP	687/3-4	100mM	505	✓
Buffer G+	NL 20	10-fold	471	✓
Eco 31 I	69/61-63	U/µL	1242	✓
T4 DNA ligase	35/14	U/µL	62	✓
Total volume		DNA: 0.55 µg/µL	56455	
Reaction tube			150 ml	
Date / Signature 12/21/00				

further addition of
Eco 31 I for 2 hours.

500µl 69/60

12/22/00 2:18 pm -
4:11 pm

15 - 20 hours at 37°C

Start of Incubation (date / time): 12/21/00 2:02 pm

End of Incubation (date / time): 12/22/00 10:08 am

3. Test restriction digest of T7-polymerase (SPO-P-11 Version: _____)

	Number	Concentration	Volume [µL]	
			Ligation	T7 restriction
Reaction batch	----	----	2	2
Water	----	----	16.2	16
Buffer G+	NL 20	10-fold	1.8	1.8
T7-Polymerase	71/1	10U/µL	-	0.2
Total volume			20	20
Time [hours]			2	2
Date / Signature 12/22/00				

1% agarose gel: (5 µL per track)

Ligation acceptable?

☐ yes☐ no

T7-polymerase digest positive?

☐ yes☐ noProceed with T7-polymerase digest?
(Step 4)☒ yes☐ no

Date / Signature

Remarks see reverse:

12/22/00

EXHIBIT E

